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Noncovalent Protein and Peptide Functionalization of Single-Walled Carbon Nanotubes for Biodelivery and Optical Sensing Applications

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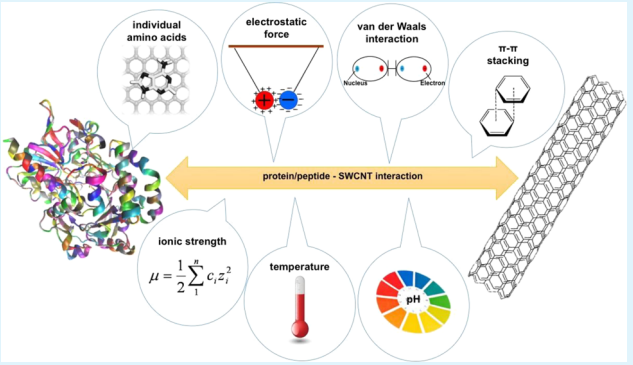
ABSTRACT: The exquisite structural and optical characteristics of single-walled carbon nanotubes (SWCNTs), combined with the tunable specificities of proteins and peptides, can be exploited to strongly benefit technologies with applications in fields ranging from biomedicine to industrial biocatalysis. The key to exploiting the synergism of these materials is designing protein/peptide-SWCNT conjugation schemes that preserve biomolecule activity while keeping the near-infrared optical and electronic properties of SWCNTs intact. Since sp^2 bond-breaking disrupts the optoelectronic properties of SWCNTs, noncovalent conjugation strategies are needed to interface biomolecules to the nanotube surface for optical biosensing and delivery applications. An underlying understanding of the forces contributing to protein and peptide interaction with the nanotube is thus necessary to identify the appropriate conjugation design rules for specific applications. This article explores the molecular interactions that govern the adsorption of peptides and proteins on SWCNT surfaces, elucidating contributions from individual amino acids as well as secondary and tertiary protein structure and conformation. Various noncovalent conjugation strategies for immobilizing peptides, homopolypeptides, and soluble and membrane proteins on SWCNT surfaces are presented, highlighting studies focused on developing near-infrared optical sensors and molecular scaffolds for self-assembly and biochemical analysis. The analysis presented herein suggests that though direct adsorption of proteins and peptides onto SWCNTs can be principally applied to drug and gene delivery, *in vivo* imaging and targeting, or cancer therapy, nondirect conjugation strategies using artificial or natural membranes, polymers, or linker molecules are often better suited for biosensing applications that require conservation of biomolecular functionality or precise control of the biomolecule's orientation. These design rules are intended to provide the reader with a rational approach to engineering biomolecule-SWCNT platforms, broadening the breadth and accessibility of both wild-type and engineered biomolecules for SWCNT-based applications.



KEYWORDS: *single-walled carbon nanotube (SWCNTs or SWNTs), multi-walled carbon nanotubes (MWCNTs or MWNTs), biomolecules, nanoparticles, bioconjugation, optical nanosensors, near-infrared (nIR) fluorescence*

1. INTRODUCTION

Since the first characterization of individual single-walled carbon nanotube (SWCNT) fluorescence in 2002,[1] the following decade has seen an over 600% surge in SWCNT-based research. SWCNTs have proven to be among the most versatile materials today, enabling a breadth of novel hybrid materials in fields spanning healthcare,[2−4] molecular biology,[5,6] energy,[7,8] and catalysis.[9] A large fraction of this recent research has focused on exploiting intrinsic SWCNT optical properties as a means of signal transduction in bio-optical devices.[10−12] The indefinite photostability, combined with the single-molecule sensitivity limits and optical transparency of biological tissue to near-infrared fluorescence, substantiates the increased use of SWCNTs in sensing platforms for a nearly limitless range of applications.[11−15]

By virtue of their highly hydrophobic nature, one of the primary challenges in engineering carbon nanotube-based materials such as SWCNTs and the closely related multi-walled carbon nanotubes (MWCNTs) is overcoming their pronounced tendency to strongly self-associate in water and form thick, insoluble, toxic aggregates. Both covalent and noncovalent sidewall surface functionalization can be used to disperse nanotubes in aqueous solutions to enhance their biocompatibility. However, in the case of SWCNTs, the aggregates demonstrate diminished fluorescence[16] that compromises not only the use of SWCNTs for optical applications, but also nanotube dispersibility in a manner that hinders their interaction with biological components under physiological conditions. Because covalent functionalization perturbs and even diminishes SWCNT optical properties,[17] an essential element in the design of optical SWCNT-based devices is the noncovalent conjugation of SWCNTs. Noncovalent function-

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alization of nanotubes[18] has already been rigorously developed for MWCNTs using linker molecules[19,20] and polymers.[21] SWCNT functionalization has been similarly achieved with a variety of wrappings such as synthetic[22] and biopolymers[23−26] (e.g., DNA, RNA oligonucleotides), peptides,[27,28] and proteins.[29−32] These wrappings can stabilize SWCNT dispersions in water without altering the extended π-conjugated system of the nanotube.[33]

In addition to improved dispersivity, noncovalent wrappings can also impart the SWCNT with secondary characteristics such as molecular selectivity. In this regard, protein- and peptide-based conjugation strategies are particularly advantageous for the development of SWCNT devices that require specific molecular recognition or biocharacterization capabilities.[34−36] Optical protein-SWCNT technologies developed using these noncovalent conjugation strategies include, for example, enzyme-based optical biosensors for the label-free detection of target analytes.[14,37] In addition to bioanalyte sensing applications, the high surface area-to-volume ratio and uniform structural integrity of the surface allow the nanotube to behave as a scaffold for protein and peptide immobilization[34,38] for drug delivery applications. Nanotube fluorescence in this regard has been used to monitor protein self-assembly[39] and activity.[40]

Most noncovalent immobilization techniques can be broadly categorized into two groups: (i) purely adsorptive and (ii) hybrid approaches (Figure 1). The purpose of this review article is to present an overview of the various conjugation strategies in each of these categories. We highlight experimental applications relevant to each strategy and discuss the key underlying forces that govern the nature of the protein/peptide-SWCNT interactions. These forces contribute to characteristics such as adsorption efficiency, biomolecule orientation, multicomponent self-assembly mechanisms, stability, protein activity, and, ultimately, SWCNT fluorescence response. This understanding is used to identify and optimize appropriate conjugation strategies for different applications.

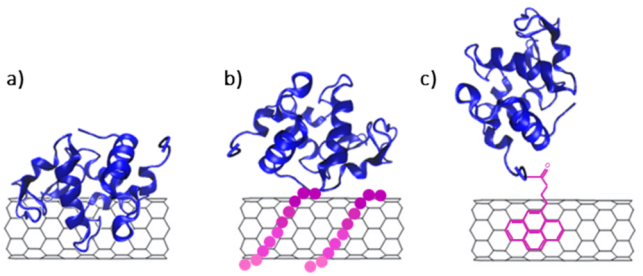

Figure 1. Overview of approaches for noncovalent protein (blue) conjugation to a SWCNT (black): (a) nonspecific physical adsorption of a protein to a SWCNT, (b) hybrid conjugation through covalent attachment to noncovalent wrappings (magenta), and (c) protein binding to heterobifunctional linker molecules (magenta) adsorbed onto SWCNTs.

2. NONSPECIFIC PROTEIN AND PEPTIDE ADSORPTION

A survey of the literature on protein/peptide-SWCNT conjugation shows that nonspecific adsorption, because of its simple and generally applicable procedure, is one of the most commonly used strategies for developing novel nanotube-based technologies. Protocols often consist of an ultrasonication step in which carbon nanotubes are individually debundled in solutions containing the desired protein/peptide, followed by ultracentrifugation and filtration to remove remaining aggregates and unbound molecules. Since direct ultrasonication of the protein may result in denaturation, an alternative approach to conjugating SWCNTs is to suspend the SWCNTs in surfactant and then dialyze the surfactant in the presence of the protein/peptide to assist in biomolecule adsorption upon gradual removal of the detergent.[37,41]

As reported by Andrade et al.,[42] every protein presents its own "unique molecular personality", and, depending on the protein structure and scope of application, different parameters need to be taken into account to optimize assembly on the SWCNT surface. The spontaneous association of proteins with SWCNTs occurs when the Gibbs binding free energy is negative ($\Delta G < 0$).[43] In contrast to small gas particles, whose symmetric, rigid structures typically give rise to Langmuir-type adsorption behavior,[44,45] proteins often undergo additional complex phenomena such as conformational rearrangements, changes in orientation, aggregation, and lateral interactions that further complicate the study of their interfacing with substrates.[46] Stable dispersions are the result of electrostatic or steric repulsion provided by the coating molecules as well as favorable van der Waals, hydrophobic, and π-stacking interactions at the SWCNT interface dominating over the strong van der Waals attractive forces between the carbon nanotubes.[30,47]

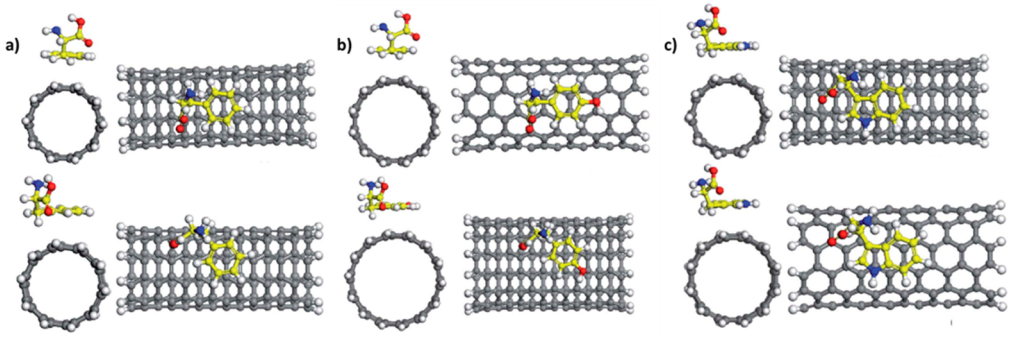

Figure 2. Most stable (a) Phe/(5,5)-SWCNT (top and bottom); (b) Tyr/(6,6)-SWCNT (top), Tyr/(7,7)-SWCNT (bottom); (c) Trp/(6,6)-SWCNT (top) and Trp/(7,7)-SWCNT (bottom) complexes in two different views, the front view (left side) and top view (right side). Reproduced with permission from ref 50. Copyright 2011, Royal Society of Chemistry.

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When considering the individual contribution of specific amino acid residues on the total free energy of protein and peptide binding, aromatic amino acid residues are shown to exhibit the strongest affinity for SWCNTs in terms of adsorption efficiency and interaction energy.[48] Through combined Monte Carlo modeling and *in vitro* selection techniques such as phage display, Wang et al.[49] engineered polypeptides capable of recognizing the surface of SWCNTs using consensus binding sequences containing motifs rich in aromatic side chains at specific locations. By investigating the adsorption of aromatic amino acids Phe, Tyr, and Trp onto the SWCNT surface, Wang and co-workers[50] identified $\pi-\pi$ stacking interactions between the benzene (Phe and Tyr) and indole (Trp) rings of the residues and carbon nanotubes as key interactions in the adsorption process (Figure 2). Of the aromatic residues, Trp and Phe were shown to have the highest and lowest binding affinities toward carbon nanotubes, respectively.[51] The interaction of His with SWCNTs was found to be weaker than these aromatic amino acids because of the less pronounced aromaticity of its imidazole.[48]

In addition to aromaticity, charge distribution may also contribute to amino acid-SWCNT binding. In fact, one of the most extensively studied model proteins, lysozyme (LSZ), has been shown to solubilize SWCNTs largely through charge interactions between Arg residues in the two loop regions at the SWCNT interface (Figure 3). The guanidinium group at the

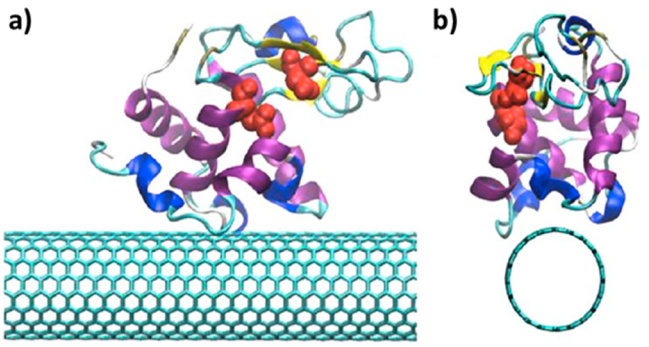

Figure 3. Interaction between the (10,10)-SWCNT and lysozyme from the (a) side and (b) front view. The catalytic residues of the protein is shown in red. Reprinted in part with permission from ref 32. Copyright 2016 American Chemical Society.

end of the Arg side chain strongly binds to aromatic moieties, with cation$-\pi$ interactions dominating on the SWCNT sidewall.[52] This explains why, in addition to peptides and proteins, positively charged homopolypeptides like poly-L-arginine (PLA) have a strong affinity for SWCNTs.[27] Monomeric amino acids mainly exist as zwitterions in aqueous environments, containing both negatively charged ($-COO^-$) and positively charged ($-NH_3^+$) groups. The protonated amine group is predicted to preferentially bind the SWCNT because protonation strengthens the polarity of the amino acid zwitterion. Previous studies have shown that the Gly zwitterion has a stronger affinity to the armchair (3,3)-SWCNT than the nonionic Gly, and the adsorption energy of Gly on the SWCNT is comparable to that observed for gas molecules.[53,54] Moreover, direct exposure of the negatively charged carboxylate and positively charged ammonium groups, which extend away from the nanotube surface and toward the aqueous solution, facilitates the solubilization of SWCNTs in water, likely providing the electrostatic repulsion that prevents SWCNT reaggregation.

Polarity, hydrophobicity, and van der Waals interactions may also aide in the protein- and peptide-based solubilization of SWCNTs. Because these interactions are considered weaker than electronic SWCNT interactions, proteins may require a significant percentage of basic, polar amino acid residues to be able to readily stabilize SWCNTs in aqueous environments (Figure 4).[30] However, proteins such as histones (HSTs),

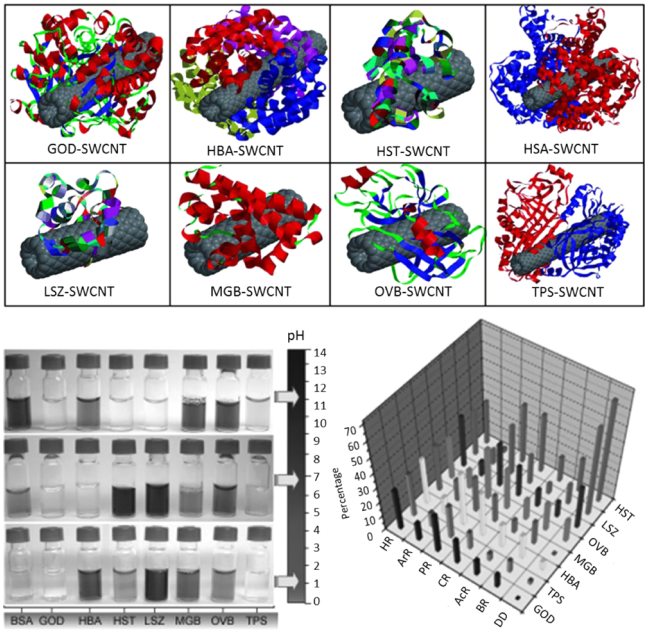

Figure 4. Top: schematic representation of protein-SWCNT complexes: glucose oxidase (GOD), hemoglobin (HBA), histone (HST), human serum albumin (HSA), lysozyme (LSZ), myoglobin (MGB), ovalbumin (OVB), and trypsin (TPS). Bottom, left: stability of the protein-SWCNT dispersions at different pH. Bottom, right: bars illustrating the degree of SWCNT debundling (DD) with respect to the percentage of hydrophobic (HR), aromatic (ArR), polar (PR), charged (CR), acidic (AcR), and basic (BR) residues in the protein. Reproduced with permission from ref 30. Copyright 2007 John Wiley and Sons.

which suspend SWCNTs through polar interactions, may solubilize SWCNTs more efficiently than proteins containing a small number of charged residues, such as glucose oxidase (GOD) or trypsin (TPS). van der Waals interactions between the protein/peptide and SWCNT are often considered relative to changes in SWCNT−SWCNT and SWCNT−water van der Waals interactions. For example, bovine serum albumin (BSA)-SWCNT dispersions are largely attributed to the unfavorable interactions between water and the protein-SWCNT interface that accompany the favorable van der Waals interaction between the BSA and SWCNT. Though the hydrophobicity of a protein may improve the protein's interaction with the SWCNT, hydrophobicity contributes to the agglomeration and precipitation of functionalized SWCNTs in aqueous environments.[55] The formation of these agglomerates may be detrimental to *in vivo* applications where SWCNT bundling has been shown to result in both cellular and organ toxicity.

Protein adsorption is characterized not only by the individual amino acid contributions discussed above, but also by the secondary and tertiary protein structure. Because of their

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diverse three-dimensional structures, proteins possessing similar distributions of specific residues can interact with SWCNTs in a very different manner. As reported by Matsuura and co-workers, proteins such as papain, pepsin, LSZ, and BSA, which contain similar distributions of hydrophobic, aromatic, and polar residues in the primary sequence, demonstrate considerably distinct binding behaviors.[56] Analogously, Ge and co-workers studied the adsorption behavior of blood proteins, namely bovine fibrinogen (BFG), gamma globulin (Ig), transferrin (Tf), and BSA, onto SWCNTs.[57] Although these proteins share a similar percentage of hydrophobic residues, the authors identified a competitive adsorption order, BFG > Ig > Tf > BSA, that positively correlates with the number of contact residues and the binding surface area of the proteins.

2.1. Engineering Protein/Peptide Dispersivity by Tuning Environmental Contributions. Sensing or delivery applications often require a specific protein to be immobilized onto the SWCNT surface. The rational design of glucose sensors, for example, may restrict the selection to glucose sensitive proteins such as GOD and glucose binding proteins where the distribution of aromatic, charged, polar, and hydrophobic residues is largely predetermined. The wild-type distribution of residues may not be suitable for solubilizing SWCNTs through the interactions described above. In such cases, exogenous factors, such as SWCNT morphology and the physiochemistry of the surrounding solution, may be tuned to facilitate solubilization.

The diameter, length, and chirality of the SWCNT have been shown to play a significant role in the amino acid-SWCNT interaction. For example, amino acids show a higher tendency to adsorb on chiral and zigzag SWCNTs compared to armchair SWCNTs.[58] This observation has been attributed to the higher aromaticity of the zigzag and chiral SWCNTs, which, as discussed above, increases binding affinity. Furthermore, increasing contact area by, for example, increasing the diameter or length of the SWCNT enhances the adsorption energy.[50] Density functional theory (DFT) and Møller−Plesset second-order perturbation theory (MP2) calculations have shown that the most preferable geometrical orientation for all aromatic rings is parallel to planar graphene sheets with distances around 3.21, 3.33, 3.34, and 3.50 Å for His, Phe, Tyr, and Trp rings, respectively.[59] Overall, the interaction between the amino acids and graphene was found to be stronger than that for SWCNTs, suggesting that increased curvature disrupts the binding interaction.[60,61] These calculations have been supported by an observed monotonic decrease in BSA adsorption capacity with increasing nanomaterial curvature (Figure 5).[62]

In addition to noncovalent interactions between the biomolecule and nanotube, environmental conditions such as ionic strength and pH may also alter protein adsorption. High ionic strength solutions such as cell culture media may subject protein-SWCNT complexes to reaggregation or a reduction in dispersion yield.[63] Variations in pH may change the net charges of the amino acid residues, strongly influencing the charge interactions that contribute to protein adsorption.[64] According to Derjanguin, Landau, Verwey, and Overbeek (DLVO) calculations,[65,66] a deficiency in the net charge around the isoelectric point (IEP) of the protein results in attraction between suspended SWCNTs, resulting in flocculation. In the case of BSA, different structural conformations are known to depend on pH, shifting from an elongated form (E form) under acidic conditions (pH < 4), to a bulky and packed conformation (N form) near isoelectric values (4 < pH < 8),

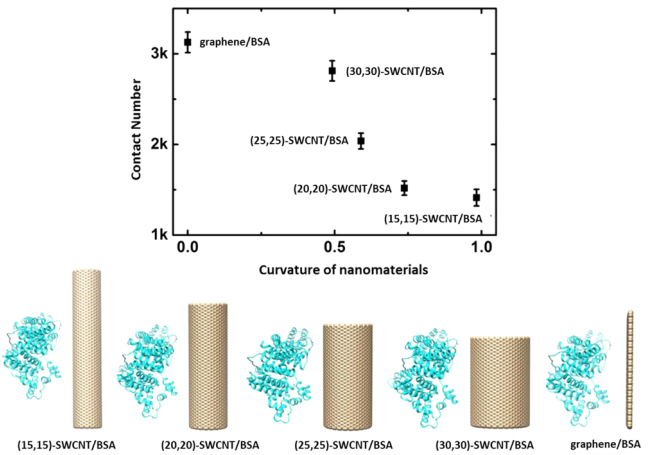

Figure 5. Contact number versus the surface curvature (defined as $c = 1/r$, where r represents the radius of the SWCNT) of carbon-based nanomaterials. Reprinted in part with permission from ref 62. Copyright 2015 Nature Publishing Group.

to a looser yet bulky conformation (B form) at basic conditions (pH > 8).[67] SWCNT dispersion was shown to increase with the bulkiness of the protein conformation and with electrostatic charges for a given amount of bulkiness. In contrast to BSA-SWCNT, LSZ-wrapped SWCNTs show a strong pH-switching effect that results in the instability of these complexes at pH values near the IEP of the protein. In the case of LSZ, protonated amine interactions on the sidewalls of the carbon nanotube dominate at pH values below the IEP of the protein, whereas negative amine adsorption prevails at higher values. According to DLVO theory, the electrostatic repulsive forces at lower and higher pH stabilize the complexes in aqueous milieu. In contrast, the deficiency of charges around the IEP results in pronounced flocculation in the system.

This ability to tune the agglomeration state of SWCNTs by altering the amino acid composition, combined with the associated changes in photoluminescence, offers a promising platform for developing biological pH sensors in addition to monitoring the conformational state of a protein. The drop in SWCNT photoluminescence at pH between 8 and 11 and its recovery at pH < 8 and pH > 11 can be used to monitor the pH-dependent aggregation state of the LSZ-SWCNT complex. These observations suggest that the stabilization and dispersion mechanisms of protein-SWCNT complexes are protein dependent; while electrostatic interactions play a dominant role in stabilizing LSZ-SWCNT, protein conformation modulates the stability of BSA-SWCNT complexes even at the IEP, where electrostatic repulsion is negligible.

In addition to pH and ionic strength, protein concentration may also substantially affect the quality of the dispersion. Bundle-free or isolated SWCNTs typically exhibit sharp peaks in the visible and near-infrared regions of the optical absorbance spectra due to the van Hove transitions of metallic and semiconducting SWCNTs.[30] Broad, weak absorbance peaks often indicate the presence of aggregated SWCNTs, since the van der Waals interactions between stacked tubes disturb the electronic structure of SWCNTs.[1] Several studies have identified a protein:SWCNT threshold (e.g., 5:1 for BSA:SWCNT) below which a high yield but poor dispersion quality is obtained and above which a higher dispersion, but smaller yield, is obtained.[63] The unexpected decrease in yield with increasing protein concentration can be explained by the

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protein-mediated depletion attraction phenomenon. Depletion attraction is an entropically driven aggregation force that occurs in colloidal systems containing particles of different sizes. These forces arise from a balance between the translational and orientational entropy of SWCNTs and the translational entropy of the protein.[67] The presence of excess protein induces attractive intertube forces that reduce the dispersed SWCNT concentration. When additional BSA is added to BSA-functionalized SWCNTs, the free BSA molecules do not cause SWCNT flocculation. In contrast, the same amount of additional protein added prior to sonication results in SWCNT coagulation. It is believed that the strong BSA-SWCNT binding energy compensates for the depletion forces acting when excess BSA is added after sonication. Horn et al. (2016) observed this protein concentration dependency in the case of LSZ-SWCNT.[68] At low LSZ concentrations, a strong van der Waals attraction between the nanotubes dominates, resulting in SWCNT aggregation. However, at higher LSZ:SWCNT ratios, an abrupt transition to an aggregated state is observed, with the loss of SWCNT mixing entropy compensated by an increase in the LSZ translational entropy. Such a transition increases the steady shear viscosity of the solution, and the attractive interactions induce reaggregation of the SWCNTs only when the LSZ concentration is sufficiently large.

2.2. Adsorption Effect on Protein Fold and Activity for Delivery Applications. Protein adsorption may perturb protein structure, or even functionality, a drawback that may pose serious limitations for sensing applications that require a protein's molecular recognition capabilities. In 2004, Karajanagi and co-workers became one of the first groups to study the influence of SWCNTs on the functionality of enzymes such as α-chymotrypsin (CT) and soybean peroxidase (SBP), demonstrating that while SBP retained up to 30% activity upon adsorption, CT retained only 1% activity. The diminished activity observed for CT was largely attributed to extensive perturbation in the secondary structure of the protein. The disparity in the adsorption effects on protein activity was rationalized from a structural standpoint; SBP contains 19 hydrophobic residues on the surface of the protein that may participate in favorable nanotube interactions. Since these are surface residues, the interaction is not believed to compromise the integrity of the active site that lies on the interior of the protein. On the other hand, CT needs to partially unfold in order to effectively interact with the nanotube surface with its inner residues, a phenomenon that has also been observed for human blood proteins[57] as well as LSZ[69,70].

Though SWCNT adsorption may contribute to protein unfolding, under strongly denaturing environments, SWCNT adsorption may actually enhance protein stability.[71] SBP, for example, which undergoes rapid denaturation at 95 °C, is 10-fold more stable at this elevated temperature once adsorbed onto SWCNTs. It has been hypothesized that the stabilization results from the peculiar curvature of SWCNTs, which suppresses unfavorable protein−protein lateral interactions. Moreover, the increased peptide-backbone conformational freedom at elevated temperatures has been shown to facilitate protein association with SWCNTs.[72] On the other hand, lack of elevated temperature control during sonication, which is frequently employed as a means of nanotube debundling and functionalization, is likely to result in protein denaturation, thus deeply perturbing its adsorption behavior.[56,73] Varying sonication times and power can alter protein secondary structure with dramatic consequences for biological applications, as conformational changes may result in the complete loss of protein activity.[63,74]

3. NONSPECIFIC ADSORPTION OF NATURAL AND SYNTHETIC MEMBRANE FRAGMENTS

The examples discussed thus far largely hold for soluble proteins that are capable of maintaining their fold in the absence of solubilizing surfactants or lipids. However, many vital proteins function only in a bilayer membrane environment and thus often require additional considerations to provide a viable route for their functional coupling to the SWCNT surface. Artyukhin and co-workers,[75] for example, modified the SWCNT surface with a hydrophilic polymer cushion layer obtained by layer-by-layer self-assembly of alternating oppositely charged polyelectrolytes such as poly(diallyldimethylammonium chloride) (PDDA), polystyrenesulfonate (PSS), and poly(allylamine hydrochloride) (PAH). This derivatization process was followed by the formation of lipid bilayers through vesicle fusion, yielding a synthetic membrane that can be used to support membrane proteins such as the anthrax protective antigen fragment PA63.

Photoresponsive membrane proteins present an additional constraint that requires the protein to be orientated in a manner that ensures effective charge transfer to the SWCNT.[8,39] In 2005, Bradley et al.[76] integrated the cell membrane of *Halobacterium salinarum*, which harbors photosensitive bacteriorhodopsins (BRhs) that are largely uniformly orientated in the host membrane, in a carbon nanotube-based transistor. Five years later, a sodium cholate suspension-dialysis method was used by Bertoncini et al.[77,78] to noncovalently immobilize natural purple membranes consisting of lipids and BRhs on the sidewalls of SWCNTs, and the resulting complexes showed pH-dependent energy transfer from the BRh to the SWCNTs with efficiencies around 94%. The hydrophobic forces between the α-helices and the sidewalls of nanotubes dominate this interaction, and the relatively weak nature of these forces resulted in minimal conformational, and consequently activity, perturbation. Ham and co-workers[8,39] relied on the use of synthetic lipid bilayer discs called nanodiscs to encapsulate and stabilize purified light-harvesting reaction centers isolated from the photosynthetic purple bacterium, *Rhodobacter sphaeroides*. In this approach, sodium cholate-suspended carbon nanotubes were dialyzed in the presence of phospholipids, membrane scaffold proteins, and reaction centers. Upon removal of the surfactant, the remaining components spontaneously self-assemble, forming nanodiscs that unidimensionally align along the length of the nanotube with individual proteins embedded within the bilayer discs (Figure 6). The hole injection site, which is located in a relatively hydrophobic domain of the protein, is hypothesized to be directed toward the sidewalls of the SWCNTs, with the

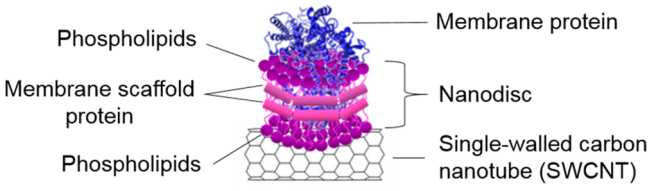

Figure 6. Schematic representation of a synthetic lipid bilayer disc, or nanodisc, embedding a reaction center protein on the surface of a SWCNT.

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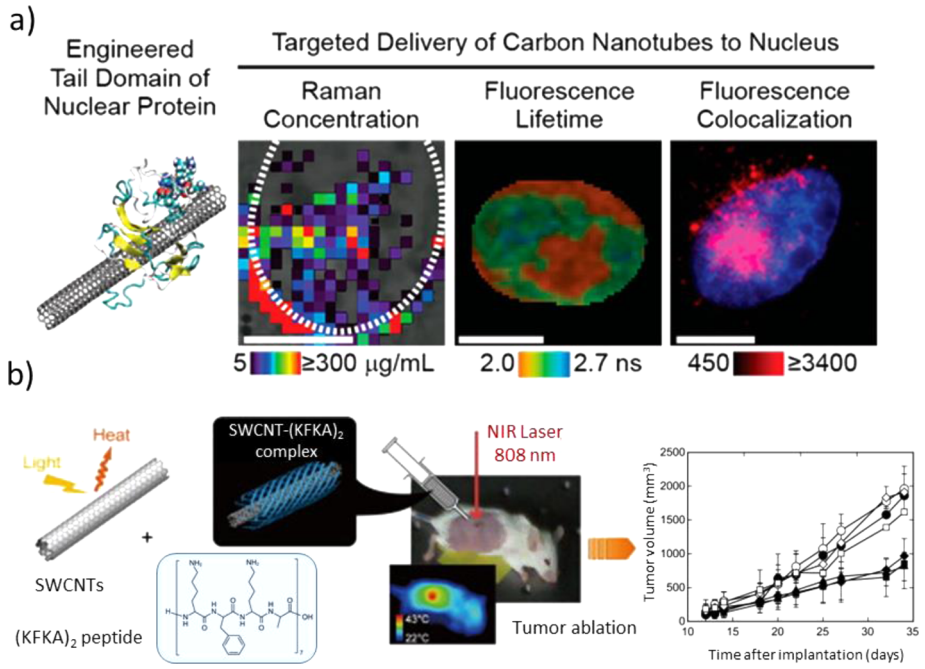

Figure 7. (a) Targeted nuclear delivery of SWCNTs in HeLa cells is facilitated by noncovalently attaching engineered nuclear proteins LB1. (b) Peptide-SWCNT composites are injected in tumor cells and used in photothermal cancer therapy. Reproduced with permission from refs 93 and 94. Copyright 2016, American Chemical Society. Copyright 2013 Elsevier.

hydrophilic globular portion of the protein exposed to the external solution.

4. HYBRID APPROACH: COVALENT ATTACHMENT TO NONCOVALENT WRAPPINGS

The enzymatic activity and intrinsic selectivity of most proteins often require the nearly complete retention of their native fold,[79] and, as discussed above, the hydrophobic surface of SWCNTs provides a fundamentally divergent environment that may perturb structure and functionality in a protein-specific manner.[80] An alternative bioconjugation strategy that offers more control over protein orientation while circumventing the structural perturbation caused by direct adsorption is the hybrid noncovalent/covalent approach (Figure 1b, c). In one approach, the SWCNT is noncovalently covered by a synthetic or biological polymer that is covalently conjugated to the protein (Figure 1b). For example, Kim and co-workers[81] functionalized the SWCNT surface with phospholipid polymers containing carboxylated poly(ethylene glycol) (cPVA), which was covalently tethered to D-luciferase through a carbodiimide-activated cross-linking reaction to create an optical ATP sensor. ATP is selectively consumed by luciferase in a two-step bioluminescent reaction that quenches SWCNT fluorescence in living cells, enabling spatial cellular ATP detection with a detection limit of 240 nM. A similar conjugation approach was used to immobilize glucose binding protein (GBP), which is a periplasmic binding protein that undergoes a significant conformational change upon binding its target analyte, glucose. This platform was used to create a reversible optical glucose sensor for continuous blood sugar monitoring.[40,82] This conjugation strategy relies on the covalent conjugation of a Lys residue on the GBP to carboxylated poly(vinyl alcohol) (PVA)-wrapped SWCNTs through amide bond formation. Compared to GBP physisorption, the additional degrees of rotational and translational freedom allow the protein to behave as a nanoscale actuator, modulating the fluorescence signal in response to glucose. In addition to PVA, other polymers that have been used to conjugate SWCNTs include poly ethylene oxide (PEO) derivatives, which have been shown to reduce the nonspecific adsorption of proteins while enabling the selective recognition of target molecules of interest,[83] and pluronic P103, a triblock copolymer with hydrophilic PEO segments that leave the SWCNT surface with hydrophilic, charge-neutral characteristics that suppress nonspecific binding to the SWCNT surface. These polymers have been key in developing highly specific electronic sensors for the detection of clinically relevant biomolecules such as antibodies.[84]

Heterobifunctional cross-linking agents may be used as an alternative conjugation approach (Figure 1c). The cross-linking agents often contain an aromatic moiety that can interact with the π-network of SWCNTs and at least one functional group that can be used to selectively bind desired protein target residues, such as Lys or Cys. The amino acid-specific functional group helps control protein orientation by limiting the total possible number of accessible sites on the protein. The most common commercially available derivative used is 1-pyrenebutyric acid *N*-hydroxysuccinimide ester, which contains terminal groups that are highly reactive to nucleophilic substitution by primary and secondary amines that are abundantly present on the surface of most proteins. This technique enables the immobilization of a wide range of biomolecules on the sidewalls of SWCNTs, ensuring both high specificity and efficiency, as demonstrated by Chen and co-workers with ferritin, streptavidin, and biotinil-3,6-dioxaoctanediamine[85] and by Besteman et al. with GOD.[86] Carbon nanotubes were also noncovalently functionalized with 1-aminopyrene and used for immobilizing laccase via a glutaraldehyde cross-linking reaction.[87] Surfactant molecules may also behave as chemical linkers, adsorbing to the SWCNT surface primarily through hydrophobic interactions. Suma and co-workers, for example, used Triton X-100-functionalized nanotubes to immobilize a recombinant deoxygenase that catalyzes the ring cleavage of

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various aromatic hydrocarbons.[88] The conjugated enzyme demonstrated increased enzyme stability with varying pH, temperature, and ionic strength, maintaining activities as high as 60−79%.

Fusion proteins with terminal-linked peptides may also enable orientation-controlled protein conjugation while minimizing the interfacial interaction with the polyaromatic surface of the SWCNT. Chen et al.[89] conjugated carbonic anhydrase to the SWCNT surface by linking the C-terminus of the protein to a specific peptide sequence that has been screened by phage display to possess a very high binding affinity toward SWCNTs.[90] This approach not only improved the binding efficiency of the carbonic anhydrase by a factor of 20, but also largely retained undisrupted protein structure.

5. ENGINEERING RULES FOR BIOMOLECULE-SWCNT APPLICATIONS

The last ten years have seen significant advancements in biomolecule immobilization strategies that have improved the accessibility of previously intractable applications, particularly in the biomedical field. Depending on the importance of parameters such as protein orientation, protein activity, SWCNT fluorescence sensitivity, and SWCNT dispersion, the different conjugation strategies presented in this review offer a modular approach to engineering nanohybrid complexes for specific applications.

Because of their simple and generally reversible nature, conjugation methods based on nonspecific adsorption have been widely employed for delivery and cancer treatment applications. The high aspect ratio and surface area of SWCNTs, combined with the increased biocompatibility of protein/peptide-SWCNT complexes (e.g., no immune or acute inflammatory responses[57,91]), favor stable dispersions that are beneficial as alternative multifunctional biological transporters for oligonucleotide and drug delivery,[55] *in vivo* subcellular targeting and imaging,[91−93] or near-infrared light-based therapy of cancer[94] (Figure 7). Significant advancements in both the experimental and computational fronts over the past several years have helped elucidate the contributions of not only individual amino acid residues and larger protein domains, but also factors such as SWCNT topology on the adsorption process. These principles have been used to design amphiphilic polypeptides containing cationic groups that can efficiently bind nucleic acids, allowing SWCNTs to behave as efficient gene delivery scaffolds.[55] Targeted nuclear delivery of SWCNTs in HeLa cells has been achieved by noncovalently adsorbing engineered nuclear proteins, such as the tail domain of lamin B1 (LB1) that is capable of penetrating the cell membrane (Figure 7a). LB1's relatively small size (molecular weight of 22 kDa) and hydrophobic core are ideal for dispersing nanotubes. Simulations have shown that the lowest energetic configuration is achieved when the SWCNT is positioned in the central proteic core, which is possible after protein unfolding and refolding around the tube during sonication or when the LB1 laterally interacts with SWCNT. Since the nuclear core is positioned away from the nanotube, adsorption does not significantly compromise the targeted delivery.[93] Using a combination of aromatic, charged, and nonpolar residues, peptide engineering has also been used to disperse SWCNTs for photothermal cancer therapy (Figure 7b). Near-infrared irradiation of peptide-functionalized SWCNTs incubated with colon 26 and HepG2 cells resulted in significant cell damage, and irradiation of colon 26 tumors injected with the SWCNTs showed rapid temperature increases and suppression of tumor growth.

SWCNT intrinsic near-infrared fluorescence, which is preserved after protein physisorption, also enables real-time *in vivo* tracking of the molecular carriers, allowing improved elucidation of the main delivery pathways in eukaryotic cells. BSA-dispersed SWCNTs, for example, are readily taken up by human cells, showing distinct subcellular localizations.[91] The observed distribution to and around the nucleus with no significant harmful cellular effects suggests possible applications in gene delivery and selective targeting. The same scaffolds have also been applied for *in vivo* imaging of *Drosophila* larvae, allowing a more comprehensive study of subcellular trafficking and biodistribution of nanoparticles in intact living organisms.[92]

When long-lasting direct protein adsorption on SWCNTs is desired, particularly for difficult uptake pathways, exogenous factors can be used to prolong dispersion stability. Since parameters such as pH and ionic strength are largely controlled by the cellular environment, the strategic selection of SWCNT chirality, in addition to the amino acid sequence, become the primary means of tuning dispersivity. As discussed above, previous studies have shown an overall enhancement in biomolecule binding with increasing SWCNT diameters, and immobilizing the protein or peptide on large diameter SWCNTs may improve stability. Although precise SWCNT diameter separation remains an ongoing area of active research, multiple established protocols are available for biasing the distribution of SWCNTs toward large diameter tubes.[23,95−97]

The aforementioned examples benefit from several key characteristics that are conducive only to physisorption applications. For example, LB1 was shown to undergo significant structural changes on the SWCNT surface that may be deleterious to sensing or delivery applications that require preserved protein activity. Further, though an adsorbed conformation with a global energy minimum is favored, proteins may also assume conformations that reflect a local energy minimum, especially under kinetically limiting adsorption conditions. These conditions would introduce conformational heterogeneity to the solution mixture that limits its use in sensing or bioelectronic devices requiring a precise protein orientation on the tube surface.[98,99] Some applications require nearly wild-type protein sequences that yield poor SWCNT dispersivity even for large diameter SWCNTs, and constraints in the number and location of aromatic or charged amino acid substitutions may limit the solubility of the complex. Since environmental factors such as pH and ionic strength may also alter adsorption, applications that require the exposure of SWCNT complexes to a wide range of *in vivo* conditions are particularly challenging for protein-SWCNT complexes with limited bioengineerability. Considering that the stability of these dispersions is limited by the gradual loss of physisorbed proteins as the solution moves toward the formation of the more thermodynamically favored SWCNT bundles,[8] physisorbed complexes are often better suited for short-term applications.

In cases where a protein can only function when embedded in a membrane bilayer, either native membrane fragments or synthetic biomimetic membranes can be interfaced with the SWCNT to ensure that the protein integrity remains intact upon adsorption. This approach is hypothesized to promote more uniform control of protein orientation, which is extremely important for specific sensing or bioelectronic devices. Although this strategy offers one solution to stabilizing the

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membrane protein structure, the versatility of these platforms is limited to membrane proteins of specific sizes and geometries. Physisorption places proteins in close proximity to the hydrophobic SWCNT surface, which, depending on the nature of the biomolecule, may compromise the protein's structure and functionality.[80,100] The fewer degrees of freedom available to an adsorbed protein, combined with suboptimal orientation or adsorption-induced conformational alterations, may result in loss of protein specificity or activity.[80] The use of natural membrane fragments for suspending SWCNTs offers a much more direct approach to immobilizing membrane proteins, circumventing the need to reconstitute a synthetic membrane and benefiting from an inherent protein environment that has been optimized by nature. However, as is the case with purified protein adsorption, one drawback of using natural membrane fragments is the heterogeneity of nonspecifically adsorbed complexes and nonuniform protein distribution. Although natural and synthetic membranes offer one solution to stabilizing membrane protein structure in an oriented monolayer, the versatility of these self-assembly platforms is limited to membrane proteins of specific sizes and geometries. Self-assembly parameters such as dialysis rate, phospholipid composition, and membrane scaffold protein length would need to be optimized for proteins that have not already been integrated into this platform.

Hybrid conjugation approaches offer a more versatile, albeit more complex, conjugation approach to immobilizing proteins. Covalent protein functionalization from an individual site increases the degrees of freedom compared to complete adsorption, helping to minimize structural perturbation caused by direct adsorption. These approaches are especially suitable for SWCNT-based sensing applications that benefit from the protein's increased conformational freedom. In such applications, the protein's conformational change upon binding specific analytes may modulate the near-infrared fluorescence of SWCNTs, enabling the optical detection of target molecules such as glucose[40] and ATP[81] at very low concentrations. Some sensing applications may also require coating SWCNTs with synthetic polymers or surfactants prior to protein immobilization when it is necessary to suppress or minimize nonspecific protein binding.[101] Certain *in vivo* cellular targeting applications may also require covalent protein attachment onto nanostructured vehicles. In the study performed by Neves et al., the human protein annexin V, which is known to selectively bind phospholipids expressed externally on tumor cells, was conjugated to SWCNTs using an intermediate linker.[102] This stable platform was successfully used to treat breast cancer using near-infrared photothermal therapy. In an orthogonal approach to enhancing the specificity of a cancer drug delivery system, epidermal growth factor (EGF) was attached to the SWCNT through a biopolymer chitosan wrapping and was subsequently targeted by corresponding receptors overexpressed on the surface of cancer cells.[103] Mitomycin C was also conjugated to SWCNTs using a noncovalent/covalent hybridization approach and used for antitumor drug delivery.[104] In this particular platform, an engineered peptide directly suspended the SWCNTs, and the complex was subsequently covalently conjugated to mitomycin C. Covalent conjugation was used to moderate the pH-dependent controlled release of mitomycin C after cellular uptake.

6. CONCLUSIONS AND OUTLOOK

To date, nanobiomaterials such as protein/peptide-SWCNTs have been largely developed and optimized using a materials engineering approach. Historically, most of these techniques have focused on engineering bioconjugative chemistries and tuning nanomaterial properties. However, the relatively recent advancements in gene synthesis, decreasing DNA sequencing costs, and increasing primer accessibility have all enabled a complementary, protein engineering approach that will likely play a larger role in future bioconjugative strategies. The examples described above touch upon several instances whereby site-specific mutagenesis or SWCNT-binding sequence domains are used to target protein conjugation sites. Beyond enabling bioconjugation, protein engineering allows one to not only improve protein activity in the presence of the nanoparticle, but also altogether alter protein behavior by engineering entire protein domains. Researchers are no longer confined to only interfacing proteins nature has provided; advancements in molecular biology allow them to tune or even create new proteins with unique functions. These techniques can be used to engineer proteins with improved or altered functionalities in the presence of SWCNTs. In addition to sensing analytes of interest through analyte-protein interactions, bioengineering provides a powerful platform for screening protein−protein, protein-oligonucleotide, and oligonucleotide-oligonucleotide interactions, as well as for screening protein functionality or response toward specific analytes. Existing screening approaches, such as ELISA, may benefit from sensing platforms based on intrinsic, near-infrared SWCNT fluorescence, which obviates additional labeling steps required to obtain a photometric signal. This ability to simultaneously tune protein behavior alongside the nanoparticle properties opens the doors to a whole new level of tunability with an unprecedented reach toward designing more selective, efficient, and sensitive SWCNT-based devices for a wide spectrum of applications.

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Notes

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ABBREVIATIONS

CT = α-chymotrypsin
ATP = adenosine triphosphate
Arg = arginine
BRh = bacteriorhodopsin
BFG = bovine fibrinogen
BSA = bovine serum albumin
cPVA = carboxylated poly(ethylene glycol)
Cys = cysteine
DFT = density functional theory

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DNA = DNA
DLVO = Derjanguin, Landau, Verwey, and Overbeek
EGF = epidermal growth factor
ELISA = enzyme-linked immunosorbent assay
Ig = gamma globulin
GBP = glucose-binding protein
GOD = glucose oxidase
Gly = glycine
HBA = hemoglobin
His = histidine
HST = histone
HSA = human serum albumin
IEP = isoelecric point
LB1 = lamin B1
Lys = lysine
LSZ = lysozyme
MGB = myoglobin
MWCNT = multi-walled carbon nanotube
MP2 = Møller−Plesset second-order perturbation
OVB = ovalbumin
Phe = phenylalanine
PAH = poly(allylamine hydrochloride)
PDDA = poly(diallyldimethylammonium chloride)
PEO = poly ethylene oxide
PLA = poly-L-arginine
PSS = polystyrenesulfonate
PVA = poly(vinyl alcohol)
RNA = ribonucleic acid
SWCNT = single-walled carbon nanotube
SBP = soybean peroxidase
Tf = transferrin
TPS = trypsin
Trp = tryptophan
Tyr = tyrosine

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